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REVIEW

Current trends and future prospects of biotechnological interventions through tissue culture in apple

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Abstract Apple (*Malus domestica* Borkh.), which is a widely cultivated, important economic fruit crop with nutritive and medicinal importance, has emerged as a model horticultural crop in this post-genomic era. Apple cultivation is heavily dependent on climatic condition and is susceptible to several diseases caused by fungi, bacteria, viruses, insects, etc. Extensive research work has been carried out to standardize tissue culture protocols and utilize them in apple improvement. We review the in vitro shoot multiplication, rooting, transformation and regeneration methodologies in apple and tabulate various such protocols for easy reference. The utility and limitation of transgenesis in apple improvement have also been summarized. The concepts of marker-free plants, use of non-antibiotic resistance selectable markers, and cisgenic and intragenic approaches are highlighted. Furthermore, the limitations, current trends and future prospects of tissue culture-mediated biotechnological interventions in apple improvement are discussed.

Keywords Micropropagation · Disease resistance · Apple scab · Fruit quality · Shelf life · Transgenic · Cisgenic · Intragenic

Abbreviations

BAP	6-Benzylaminopurine
IBA	Indole-3-butyric acid
IAA	Indole-3-acetic acid
GA	Gibbrellic acid
KN	Kinetin
TDZ	Thidiazuron
NAA	1-Naphthyl acetic acid
2,4-D	2,4-Dichlorophenoxy acetic acid
ABA	Abscisic acid
TIBA	2,3,5-Triiodobenzoic acid

Introduction

Apple is an important economic fruit crop widely cultivated in temperate and sub-tropical climate. It belongs to the rose family (Rosaceae) of order Rosales and class Magnoliopsida. The cultivated apple is *Malus domestica*, while its wild relatives are *M. sieversii* and *M. sylvestris* (Coart et al. 2006). It is believed to have originated from central Asia and from there spread to the rest of the world (Harris et al. 2002). China and USA are the first and second largest apple producers, respectively. The presence of Vitamin C, β-carotene, calcium, potassium, iron, magnesium, zinc, folic, malic and tartaric acids, pectins, fibers, phenolics and flavenols in the apple fruit makes it beneficial to human health and in the treatment and prevention of several diseases (Boyer and Liu 2004; Veeriah et al. 2006). Apple is a self-incompatible, cross-pollinated horticultural crop having no true to genotype seeds. It is asexually propagated by grafting the scion on rootstock. Apple cultivation requires long chilling hours to break its dormancy and is heavily dependent on climatic condition, which is of concern in this era of global warming and climate change. Besides this, numerous diseases caused by

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various fungi, bacteria, viruses, insects (as vectors), etc., dampen apple production and its trade. Control measures for these diseases mainly rely on use of fungicidal, pesticidal and insecticidal chemicals, etc., which are again of concern to consumers as well as to the environment and their excessive usage has led to emergence of resistant strains.

Apple is one of the model horticultural crops in this post-genomic era (Shulaev et al. 2008). More than 7,500 varieties of apple are known to be cultivated. The cultivars vary in size, shape, color, crispness, firmness, texture, juiciness, sweetness and nutritional value of fruit. Absence of russeting, resistance against various apple diseases, ease of shipment, short harvesting period and lengthy storage are some of the useful features of commercial cultivars. The cultivated apple is diploid ($2n = 34$), its 2C nuclear DNA content is 1.54 pg and haploid genome size is ~750 MB (Tatum et al. 2005; Shulaev et al. 2008), which is approximately same as the genome size of sorghum (~730 MB; Paterson et al. 2009) or tomato (~950 MB; Mueller et al. 2009). Although complete genome sequence is not yet available, millions of ESTs (expressed sequence tags) are publicly available (Shulaev et al. 2008; Newcomb et al. 2006; Wisniewski et al. 2008). This has led to the development of apple microarray platform, which has proved to be extremely useful in obtaining molecular insights into fruit development, ripening and apple response against biotic stresses, etc. (Phillips et al. 2008; Lee et al. 2007; Seo and Kim 2009). Several molecular markers have been identified from apple and mapped to 17 different linkage groups (Shulaev et al. 2008). These studies have boosted up the incorporation, by apple breeding programs, of qualitative and quantitative traits into commercial cultivars (Gessler et al. 2006; MacHardy 1996; Kellerhals et al. 2004). However, due to its time-consuming nature, dependence on the availability of traits/characters (to be incorporated) in parental germplasms, chance of acquiring non-useful linked traits by progenies and threat of losing their cultivar characteristics (as progenies contain mixed characters of both parents), alternatives to breeding are being investigated to engineer useful characters.

Through advancement of biotechnological tools, a particular gene and/or group of genes can be incorporated and/or their expression can be altered to incorporate new character(s) in the existing cultivar without changing other characteristics. Furthermore, genes from other plants or systems (microbes, etc.) can also be utilized to incorporate useful characters. Tissue culture plays a key role in biotechnology-mediated crop improvement and clonal propagation of plants. Due to the focused efforts of several researchers, standardized biotechnology, molecular biology and transgenic tools are available in apple (Gessler and Patocchi 2007; Aldwinckle and Malnoy 2009). In this study, we review apple tissue culture techniques and tabulate various such protocols. We also describe the utility of tissue culture-mediated biotechnological interventions for apple improvement and discuss the current trends and future prospects.

Apple micropropagation

Tissue culture has been extensively used for raising multiple clones (micropropagation) of apple rootstocks/cultivars. Besides this, it is also useful in raising virus-free planting material, cryopreservation of genetic resources, development of synthetic seeds and apple improvement through transgenics (Dobránszki and Teixeira da Silva 2010). For micropropagation, explants are surface sterilized, inoculated on culture establishment/initiation medium, multiplied on the medium mostly consisting of cytokinins and are subjected to rooting. The success of micropropagation depends on various factors such as type of explant, season during its collection, age and genotype of stock plant, carbon source, composition of culture medium, plant growth regulator (PGR), pH, culturing conditions, etc. (Dobránszki and Teixeira da Silva 2010). MS (Murashige and Skoog 1962) is the most preferred medium for micropropagation. Table 1 enlists various plant growth regulators along with optimal culture media used for apple shoot culturing. Shoot tips or nodal shoot segments are most commonly used explants for shoot culturing in apple. Shoot tip explants produced long shoots with few axillary branches, whereas nodal explants produced shorter but more axillary shoots in cv. Tydeman's Early Worcester. Early sprouting and maximum plant establishment were observed with explants collected in spring or summer than those collected in other season (Modgil et al. 1999a). BAP is the preferred cytokinin for apple shoot multiplication; however, other analogs of benzyladenine could also enhance shoot proliferation (Dobránszki and Teixeira da Silva 2010). Although sucrose is commonly used as carbon source for shoot proliferation, Yaseen et al. (2009) have observed that sorbitol can produce better caulogenic response than sucrose, glucose and mannitol in case of apple rootstocks, M9 and M26. Additives such as PG (phloroglucinol) alone at 100 mg/l enhanced shoot proliferation in MM106 (Sharma et al. 2000) and in combination with GA resulted in better shoot proliferation in Gami Almasi (Rustaei et al. 2009). The quality of light provided during shoot proliferation had been found to affect apical dominance, bud differentiation and branching pattern in MM106 and M9 (Muleo and Morini 2006, 2008). Ethylene, the gaseous plant hormone, has significant influence on various stages of

Table 1 Optimal media compositions used for apple shoot multiplication

Apple	Explant	Initiation medium	PGR + Additives	Multiplication medium	PGR + additives	Culture response	References
Cultivar							
Tydeman's Early Worcester	AB	MS	BAP 4.4 μM + GA 2.8 μM + IBA 0.5 μM + PVP 500 mg/l + AC 100 mg/l	MS	BAP 2.2 μM + KN 7.5 μM	MSH	Modgil et al. (1999a)
Gala	MST	–	–	MS	BAP 2.22 μM + IBA0.49 μM + Sucrose 3% + Agar 0.6%	SG	Montecelli et al. (2000)
Gala, Royal Gala, Jonagold	S	–	–	MS[a]	BAP 1 mg/l + KN 3 mg/l + NAA 0.1 mg/l + Sucrose 3%; Agar 0.7%; pH 5.7	SG	Sriskandarajah et al. (1990)
Fuji	S	–	–	MS	BAP 1 mg/l + IBA 0.3 mg/l + Sucrose 3% + Agar 0.8%; pH 5.8	SG	Seong and Song (2008)
Malus sieboldii genotypes	NS	MS[b]	BAP 4.4 μM + GA 0.28 μM + IAA 0.25 μM/ or with IBA 0.25 μM + AA 57 μM + Sucrose 88 μM; Agar 0.7%; pH 5.6–5.7	MS[b]	IBA 0.25 μM + BAP 4.44 μM + GA 0.28 μM + Sucrose 88 μM; Agar 0.7%	HP + SG	Ciccoti et al. (2008)
Marshall McIntosh	ST	–	–	MS	BAP 1 mg/l + GA 0.2 mg/l + IBA 0.3 mg/l; Sucrose 3%; Agar 0.7%; pH 5.6	SG	Bolar et al. (1999)
Holsteiner Cox	MST	–	–	MS	BAP 4.4 μM + IBA 0.5 μM + Sucrose 3% + Agar 0.8%; pH 5.8	SG	Degenhardt and Szankowski (2006)
Elstar	MST	–	–	MS	BAP 3.1 μM; Sucrose 3%; Agar 0.8%; pH 5.7	SG	Szankowski et al. (2003)
Rootstock							
Gami Almasi	MST	–	–	MS	BAP 1 mg/l + NAA 0.1 mg/l + GA 2 mg/l + PG 80 mg/l	HSM	Rustaei et al. (2009)
M9	S	–	–	MS[c]	BAP 4.4 μM + GA 0.29 μM + IBA 0.49 μM + 1,3,5-Trihydroxybenzene 1 mM + Sucrose 87.7 mM; Agar 0.6%; pH 5.65	MSH	Grant and Hammatt (1999)
MM106	AB	MS	BAP 1 mg/l + GA 0.5 mg/l + IBA 0.1 mg/l	MS	BAP 0.5 mg/l + GA 1 mg/l + IBA 0.1 mg/l + PG 100 mg/l	MSH	Sharma et al. (2000)
	S	–	–	MS	BAP 4.43 μM + IBA 0.5 μM + Sucrose 87.6 mM; Agar 0.6%; pH 5.8	SG	Bahmani et al. (2009)
MM111	AB + SA	MS	BAP 0.5–1.0 mg/l + GA 0.5 mg/l with or without IBA 0.05–0.1 mg/l	MS	BAP 1 mg/l + GA 0.5 mg/l	HM and LST	Kaushal et al. (2005)
M26	SA	½ MS[d]	BAP 4.4 μM + IBA 0.5 μM + GA 0.3 μM + Sucrose 2%; Agar 0.7%; pH 5.8	LM[e]	BAP 4.4 μM + IBA 2.5 μM + GA 0.3 μM	MSH	Aklan et al. (1997)

AA ascorbic acid, *AB* axillary buds, *ABM* axillary bud multiplication, *AC* activated charcoal, *ASHD* axillary shoot development, *HM* high multiplication, *HP* high proliferation, *HSM* high shoot multiplication, *INE* internode enlargement, *LS* Linsmaier–Skoog formulation, *LST* long shoots, *MS* Murashige and Skoog, *MSH* multiple shoots, *MST* microshoots, *NS* nodal segments, *PG* phloroglucinol, *PVP* polyvinyl pyrolidone, *S* shoot, *SA* shoot apices, *SG* shoot growth, *SHE* shoot elongation, *ST* shoot tips

[a] MS salts with Staba vitamins

[b] With 1.18 μM thiamine

[c] Modified MS medium with 30 μm $ZnSO_4.7H_2O$ +100 μm FeNaEDTA

[d] Modified MS medium with ½ MS macro salts, micro salts of Nitsh and vitamins of Jacquiot

[e] Lepoivre medium having ½ macro and full micro with vitamins according to Walkey

micropropagation. It has been found to negatively affect shoot proliferation in apple (Lambardi et al. 1997). The application of ethylene inhibitor aminoethoxyvinylglycine (AVG) led to improvement in shoot development and elongation in M9 and MM111 rootstocks, while treatment of ethylene precursor aminocyclopropane-1-carboxylic acid (ACC) negatively affected in vitro shoot proliferation in both of the rootstocks.

The cuttings of micropropagated shoots are inoculated as explants in rooting medium containing specific concentration of auxin (IBA being most preferred) for in vitro root induction. The rooting media compositions giving optimal rooting response in different cultivars/rootstocks of apple are enlisted in Table 2. In some protocols, the explants are first inoculated with auxin-containing rooting medium, incubated in the dark for a specific period of time, followed by shifting to auxin-free medium with 16-h photoperiod (Bahmani et al. 2009; Ciccotti et al. 2008). Some researchers prefer to dip the excised shoots in a high concentration of auxin for ~2–3 h and then shift to hormone-free medium (Kataeva and Butenko 1987; Sharma et al. 2000). Differences in free IAA level may influence in vitro rooting, as the rootstock M26 with higher level of endogenous free auxin (IAA) requires less IBA, while M9 having low endogenous free IAA needs more IBA for in vitro rooting (Alvarez et al. 1989). The treatment of BAP and GA had inhibitory effect on IBA-induced rooting in rootstock M9 cv. Jork (Pawlicki and Welander 1992). Dark treatment for few days and use of additives such as PG, activated charcoal and coumarin (1, 2-benzopyrone) was found to enhance rooting efficiency in different genotypes (Dobránszki and Teixeira da Silva 2010). The type and concentration of carbon source used also significantly affected in vitro rooting. In MM106, the medium supplemented with 90 mM sucrose produced the highest rooting percentage and maximum roots without any hyperhydricity (Bahmani et al. 2009). Ethylene is thought to negatively affect in vitro rooting in apple, as observed by Ma et al. (1998), according to whom treatment with ethylene inhibitors ($AgNO_3$, AVG, $CoCl_2$) led to enhanced rooting efficiency, root growth and also advanced root emergence in cv. Royal Gala, while treatment with ethylene precursor (ACC) resulted in delayed root emergence with decreased root growth and number. This study points to the use of ethylene inhibitors for enhancing in vitro rooting in apple; however, its role needs to be further explored in other cultivars/rootstocks. By increasing the total time spent by the culture on shooting medium, the in vitro rooting efficiency of apple was enhanced (Grant and Hammatt 1999). The strength of various salts and their composition in basal medium significantly influence in vitro rooting in apple. Reducing NH_4NO_3 strength from full to 1/4th enhanced rooting in Gala and Royal Gala, but not in Jonagold. Further reduction of its strength from 1/4th to zero imparted better rooting response in Jonagold (Sriskandarajah et al. 1990). The authors observed the highest rooting percentage on a medium containing full strength KNO_3, but no NH_4NO_3 in all tested cultivars. Standardi and Romani (1990) studied the effect of antioxidants on in vitro rooting in apple. Antioxidants such as citric acid or reduced glutathione when applied to the root initiation/elongation medium could enhance rooting percentage; while antioxidants such as diethyldithiocarbamic acid reduced glutathione, polyvinylpyrrolidone-40 or 2-mercaptoethanol had inhibitory effect on rooting when applied to the liquid induction medium. The root inducing property of *Agrobacterium rhizogenes* had been used to induce rooting in difficult to root old shoot cultures of Golden Delicious (Patena et al. 1988) and is being explored in other genotypes of apple for root induction (Radchuk and Korkhovoy 2005; Zhu et al. 2001).

Transgenics in apple

Agrobacterium-mediated transformation is the key method for raising transgenics in apple. The majority of transgenic studies are based on the use of *A. tumifaciens* strains (EHA 105 and EHA101 being most preferred); however, *A. rhizogenes* has also been utilized in some of the studies (Aldwinckle and Malnoy 2009). Various factors such as genotype, age and source of explant, period of co-cultivation with agrobacterial strain, PGRs, etc., have been found to influence the transformation efficiency of apple (Aldwinckle and Malnoy 2009). Sucrose and glucose are favorable carbon sources for apple transformation, while fructose and galactose have detrimental effects (Bondt et al. 1994). Furthermore, sorbitol in combination with sucrose or alone has also been effective in raising apple transgenics (Borejsza-Wysocka et al. 1999; Caboni et al. 2000; Szankowski et al. 2009). The co-cultivation of explants covered with a thin layer of agrobacterial suspension for 2 days in dark provided better transformation in cv. Florina (Radchuk and Korkhovoy 2005). The presence of acetosyringone (an agrobacterial virulence gene inducer) and betaine phosphate/proline (the osmoprotectants) during co-cultivation enhanced the transformation efficiency (Seong and Song 2008; James et al. 1993). Pre-cultivation of explants for few days on shoot induction medium positively influenced the transformation efficiency of apple (Bondt et al. 1994). The post-cultivation medium solidified with gelrite yielded better transformation efficiency in cv. Jonagold (Bondt et al. 1996) and rootstock M26 (Maheswaran et al. 1992; Borejsza-Wysocka et al. 1999) than the medium solidified with agar. However, agar proved a better gelling agent for efficient transformation in

Table 2 Optimal rooting media compositions for some important cultivars/rootstocks of apple

Apple	Root induction medium	Dark treatment	Rooting medium	References
Cultivars				
Tydeman's Early Worcester	LMS + IBA 1.5 μM + sucrose 1.5%	9 days	MS[a] + IBA 2.5 μM + agar 0.8% + sucrose 1.5% + AC 0.1% + PG 100 mg/l	Modgil et al. (1999a)
Gala, Royal Gala, Jonagold	–	–	½ MS[b] + IBA 2 mg/l + sucrose 3% + agar 0.6%; pH 5.7	Sriskandarajah et al. (1990)
Fuji	–	–	½ MS salts + MS vitamins + IBA 0.3 mg/l + sucrose 1.5% + agar 0.8%; pH 5.8	Seong and Song (2008)
Malus sieboldii Genotypes	Water solution (without vitamins) + IBA 25 μM + sucrose 88 mM; pH 6.59–6.60	4 days	MS[c] + agar 0.5% + sucrose 29.3 mM	Ciccoti et al. (2008)
Marshall MacIntosh	–	–	½ MS + IBA 0.2 mg/l + sucrose 3% + agar 0.7%	Bolar et al. (1999)
Rootstocks				
M.9	Lepoivre medium + IBA 12 μM + PG 1.3 mM + agar 0.7% + sucrose 3%; pH 5.2	5 days	Lepoivre medium; without IBA	Alvarez et al. (1989)
M.9 cv. Jork	Lepoivre medium + IBA 3.2 μM (For Stem Discs) and IBA 10–30 μM (For Shoots) + Riboflavin 1 mg/l	2–3 days (For Stem Discs) 5 days (For Shoots)	Lepoivre medium + IBA 3.2 μM (For Stem Discs) and IBA 10–30 μM (For Shoots) + Riboflavin 1 mg/l	van der Krieken et al. (1991)
MM106	½ MS + IBA 5 μM + 90 mM sucrose + 0.6% agar	5 days	½ MS; without auxin	Bahmani et al. (2009)
	IBA 30 mg/l (Dip for 3 h)	–	MS (solid); without auxin	Sharma et al. (2000)
MM111	1/2 MS (liquid) + IBA 0.5 mg/l		MS (solid); without auxin	Kaushal et al. (2005)
M.26	Lepoivre medium + IBA 4 μM + PG 1.3 mM + agar 0.7% + sucrose 3%; pH 5.2	5 days	Lepoivre medium; without IBA	Alvarez et al. (1989)

LMS liquid MS medium

[a] MS medium having 1/4 macro and micro salts with 0.5 mg/l thiamine

[b] 1/2 MS medium in which plants were kept for 3 weeks at 60 $\mu Em^{-2}\ s^{-1}$ light and then shifted to 20 $\mu Em^{-2}\ s^{-1}$

[c] 1/2 MS salts without vitamins

cv. Golden Delicious (Sriskandarajah et al. 1994). Bondt et al. (1996) observed that the presence of malate in post-cultivation medium significantly increased the transformation efficiency of Jonagold. Transformation and regeneration efficiency of cv. Fuji was found to be significantly enhanced by increased ethylene production following $AgNO_3$ treatment. However, increased ethylene concentration could only enhance regeneration efficiency in Gala (Seong et al. 2005).

Although a high amount of T-DNA transfer takes place in apple, low regeneration efficiency is a limiting factor for obtaining high and stable transformation in different cultivars of apple (Maximova et al. 1998). Efforts have been made to develop reproducible and efficient regeneration protocols for transgenic studies. The explant (age, genotype, type, size, orientation on culture medium, wounding), basal medium composition, combinations and concentrations of PGRs, type of culture vessel, proper ventilation, dark or light treatment, period of incubation on regeneration medium, etc., are major factors that influence the regeneration process in apple (Liu et al. 1983; Wilson and James 2003; Gercheva et al. 2009; Nacheva and Ivanova 2006; Magyar-Tábori et al. 2010). Table 3 enlists the best (optimum) culture media for apple regeneration. Regeneration can be induced either directly from explants or indirectly through an intervening callus phase (a mass of undifferentiated and unorganized cells). Young leaves are generally preferred as explant for direct regeneration in apple (Modgil et al. 1999b; Belaizi et al. 1991; Predieri and Fasolo Fabbri Malavasi 1989; Dufour 1990). However besides young leaves, vegetative shoot apices, nodal segments, seedlings, protoplasts, endosperm, etc., have also been used as explant for indirect regeneration (Caboni et al. 2000; Awan et al. 1990; Liu et al. 1983; Saito and Suzuki 1999; Shih-Kin et al. 1977). Inoculation of leaf explants with abaxial surface uppermost on culture medium provides better regeneration in apple (Magyar-Tábori et al. 2010). Pre-conditioning of leaf explants for few days on suitable liquid medium had been shown to enhance both transformation and regeneration efficiency even in the recalcitrant genotypes (Sriskandarajah and Goodwin 1998). Among various PGRs, cytokinin plays a key role in the process of cell differentiation and regeneration (reviewed in Magyar-Tábori et al. 2010). D'angeli et al. (2001) observed that the localization of free zeatin inside the tissues gets altered during callus-mediated regeneration in rootstock Jork 9. Interestingly, it has also been observed that cytokinin used during the shoot proliferation phase can influence regeneration efficiency of leaf explants. BAP and TDZ are preferred cytokinins for apple regeneration; however because of some adverse effects at higher concentrations, other derivates of BAP such as meta-topolin and benzyladenine-9-riboside are being analyzed as better alternatives. In most of the protocols, regeneration is induced under dark conditions. Liu et al. 1983 observed that explants cultured under an initial dark period produced more organogenic (caulogenic) response than those under continuous light conditions. Interestingly, the authors observed that short exposure of red light significantly suppressed adventitious shoot development in leaf explants of Golden Delicious, whereas the effect was reverted by subsequent exposure of far-red light.

With the advancement of transformation and regeneration protocols, extensive researches using indigenous or exogenous genes are being conducted to incorporate useful characters in commercial cultivars/rootstocks of apple (Bulley et al. 2007; Gessler and Patocchi 2007; Aldwinckle and Malnoy 2009). The *Vf* gene (Syn: *HcrVf*2; *Rvi*6) imparting scab disease (caused by *Venturia inaequalis*) resistance has been cloned from *Malus floribunda* 821 (reviewed in Gessler et al. 2006; Jha et al. 2009). Expression of the gene either under CaMV35S or its own native promoter imparted scab resistance in susceptible cultivars comparable to that of *Vf* containing cultivars (Malnoy et al. 2008; Szankowski et al. 2009; Joshi et al. 2009). The chitinase gene (endo or exo) of *Trichoderma harzianum* (syn. *T. atroviride*), *AMP* gene of maize and puroindoline B of wheat had been utilized to strengthen scab resistance in different apple cultivars (reviewed in Gessler et al. 2006; Jha et al. 2009). The MpNPR1-1 (ortholog of AtNPR1; Arabidopsis non-expressor of PR) overexpressing transgenic lines of apple demonstrated broad spectrum resistance against *V. inaequalis, Gymnosporangium juniperi-virginianae* (causative of cedar apple rust) and *Erwinia amylovora* (causative of fire blight) pathogens (Malnoy and Aldwinckle 2007). The maize Leaf color (*Lc*) gene expressing transgenic apple exhibited resistance to fire blight and scab diseases (Flachowsky et al. 2010). The overexpression of genes encoding attacins (A and E), cecropin analogs, T-4 lysozyme, EPS depolymerase and harpin N and silencing of leucine-rich repeat (LRR) receptor-like serine/threonine kinases (*DIPM*) gene, which interacts with *E. amylovora* DSPE pathogenicity factor, also imparted fire blight resistance (reviewed in Malnoy and Aldwinckle 2007; Gessler and Patocchi 2007). The transgenic apple expressing biotin binding (avidin or strepavidin) proteins (Markwick et al. 2003) or a proteinase inhibitor of *Nicotiana alata* (Maheswaran et al. 2007) demonstrated resistance against light brown apple moth disease. Furthermore, trypsin inhibitor encoding *CpTI* gene of cowpea and intracrystalline protein encoding cryIA(c) gene of *Bacillus thuringiensis* was explored to impart resistance against codling moth pest (Gessler and Patocchi 2007).

Transgenic approach had been implemented to make apple trees self-fertile by silencing the S-RNase gene (Broothaerts et al. 2004). The ectopic expression of

Table 3 Optimal media compositions for regeneration of different cultivars/rootstocks of apple

Apple	Explant	Regeneration medium	PGR + Additives	Dark treatment (in days)	References
Cultivars					
Gala	L	MS	BAP 22.2 μM + NAA 1.34 μM + Sucrose 87.7 mM + CX 250 mg/l	30	Montecelli et al. (2000)
	VSA	MS[a]	BAP 17.8 μM + NAA 2.7 μM + Sucrose 29.2 mM + Sorbitol 109.8 mM + CX 250 mg/l	20[b]	Caboni et al. (2000)
	L	MS	TDZ 22.7 μM + NAA 2.6 μM + Sorbitol 3% + Gelrite 0.3%; pH 5.7	14	Szankowski et al. (2009)
Royal Gala	IN; LB	N6[c]	TDZ (1/5/10) μM + Sucrose 87.6 mM + CH 300 mg/l, Agar 0.7%; pH 5.4	10	Liu et al. (1998)
Golden delicious	IS	MS	BAP 4.4 μM + TIBA 1 μM + Sucrose 3% + Agar 0.7%	–	Belaizi et al. (1991)
MacIntosh	VSA	MS[a]	BAP 17.8 μM + NAA 2.7 μM + Sucrose 29.2 mM + Sorbitol 109.8 mM + CX 250 mg/l	20[b]	Caboni et al. (2000)
	LS	MS[d]	TDZ 3 μM + BAP 12 μM + Sucrose 3% + Agar 0.75%; pH 5.7	21	Sarwar and Skirvin (1997)
Marshall McIntosh	L	N6[e]	TDZ 2.2 mg/l + NAA 1 mg/l + Sucrose 3% + Agar 3.5 g/l + Gelrite 1.2 g/l; pH 5.6	14	Bolar et al. (1999)
Fuji	P*	MS	IAA 0.1 mg/l + ABA 0.1or 1 mg/l + TDZ 2 mg/l + Gelrite 0.7%; pH 5.7	–	Saito and Suzuki (1999)
Florina	L	½ MS[f]	BAP 5 mg/l + NAA 0.2 mg/l + Sucrose 2% + Gelrite 0.25%; pH 5.6–5.8	21	Radchuk and Korkhovoy (2005)
Macspur	LS	MS[d]	TDZ 2 μM + BAP 16 μM + Sucrose 3% + Agar 0.75%; pH 5.7	21	Sarwar and Skirvin (1997)
Wijcik	LS	MS[d]	TDZ 3 μM + BAP 20 μM + Sucrose 3% + Agar 0.75%; pH 5.7	28	Sarwar and Skirvin (1997)
Elstar	L	MS	TDZ 3 μM + IBA 1 μM + Sorbitol 3% + Gelrite 0.3%; pH 5.7	14	Szankowski et al. (2009)
Holsteiner Cox	L	MS	TDZ 3 μM + IBA 1 μM + Sorbitol 3% + Gelrite 0.3%	14	Szankowski et al. (2003)
Rootstocks					
Gami Almasi	L	MS	BAP 7.5 mg/l + NAA 2 mg/l + Sucrose 3% + Agar 0.7%	10	Rustaee et al. (2007)
M9	L	MS	NAA 5.4 μM + TDZ 15 μM + Sorbitol 4%; Gelrite 2.5 g/l; pH 5.6	14	Höhnle and Weber (2007)
Jork 9	VSA; AB	MS[a]	BAP 17.8 μM + NAA 2.7 μM +Sucrose 29.2 mM + Sorbitol 109.8 mM + CX 250 mg/l	20[b]	Caboni et al. (2000)
MM106	L	MS	BAP 22 μM + NAA 5.4 μM + Sucrose 2% + Agar 0.8%	–	Modgil et al. (1999b)
M26	L	MS[g]	BAP 4.4 μM + NAA 0.5 μM + Sucrose 2% + Agar 0.7%; pH 5.7	25	Predieri and Fasolo Fabbri Malavasi (1989)
	VSA	MS[a]	BAP 17.8 μM + NAA 2.7 μM +Sucrose 29.2 mM + Sorbitol 109.8 mM + CX 250 mg/l	20[b]	Caboni et al. (2000)

AB axillary buds, *C* callus, *CH* casien hydrolysate, *CX* cefotaxime, *IN* internodes, *IS* internodal segments, *L* leaves (in vitro), *LB* leaf blades, *LS* leaf segments, *P** protoplasts from meristem-derived calli, *VSA* vegetative shoot apices

[a] MS medium without glycine

[b] After 20 days of dark treatment, cultures were shifted to auxin-free medium under light conditions

[c] N6 medium containing N6 macro salts (Chu et al. 1975), LS micro salts, 0.56 mM myo-inositol and 3 mM thiamine-HCl

[d] Modified MS medium containing MS salts, Staba vitamins, myoinositol 100 mg/l, gentamicin sulfate 10 mg/l and ascorbic acid 50 mg/l

[e] Modified N6 medium containing thiamine-HCL 1 mg/l and myoinositol 100 mg/l

[f] Modified ½ MS medium

[g] Modified MS medium containing MS salts and LS (Linsmaier and Skoog) vitamins

gibberellic acid (GA)-insensitive (*gai*) gene of Arabidopsis (Zhu et al. 2008) and downregulation of apple GA biosynthetic enzyme, GA 20-oxidase (Bulley et al. 2005), imparted dwarfing in apple. The downregulation of apple *ARRO-1* (adventitious rooting related oxygenase) gene through RNAi led to adventitious root formation in M26, suggesting its role in root formation in apple (Smolka et al. 2009). The *rolA*, *B* and *C* genes associated with the Ri plasmid of *A. rhizogenes* had been utilized to impart dwarfing in apple. The *rolA* transgenic lines demonstrate dwarf phenotype, while the *rolB* and *rolC* transgenic lines besides having dwarf phenotype also demonstrate enhanced rooting ability (Holefors et al. 1998; Zhang et al. 2006; Zhu et al. 2001). The ability of *rolB* to enhance rooting even on hormone-free medium had been successfully utilized in various transgenic studies (Welander et al. 1998; Radchuk and Korkhovoy 2005; Zhu et al. 2001).

Limitations and future prospects of biotechnological interventions in apple

The culture contaminations, browning and hyperhydricity of culture, somaclonal variations, presence of chimeric tissues, silencing of transgene and reduced vigor are some of the commonly faced problems during tissue culture-mediated biotechnological interventions. Antibiotics such as penicillin G, streptomycin, tetracyclin, rifampicin, cefotaxime and fungicides such as proclin® 300, mancozeb, triforine, myclobutanil, silver nitrate, etc., are effective in inhibiting growth of bacterial and fungal contaminants associated with apple tissue culture (Flachowsky and Hanke 2009; Nagy et al. 2005; Savela and Uosukainen 1994). Various strategies had been explored to combat the problem of excessive phenolic exudation in culture medium. Frequent subculturing of explants on fresh agarized medium, use of small-sized explants, culturing on liquid medium for a short time (3–10 days), use of antioxidants such as ascorbic acid or citric acid, or absorbents as activated charcoal, polyvinylpyrrolidone, etc., are some of such strategies (Singh 2005; Modgil et al. 1999a; Kaushal et al. 2005). Downregulation of *PPO* gene through antisense technology could reduce the browning of apple (Murata et al. 2000). Use of temporary immersion system (Chakrabarty et al. 2007), adequate carbon source (Bahmani et al. 2009), hydrolyzed agar (Marga et al. 1997), proper concentration of cytokinin (Zhu et al. 2005) and its combination with gelling agent (Höhnle and Weber 2009) were effective in reducing hyperhydricity during apple tissue culture. Interestingly, some somaclonal variants of apple have been exploited to impart useful traits such as fire blight resistance in apple cultivar Greensleeves (Chevreau et al. 1998; Donovan et al. 1994) and enhanced resistance to *Phytophthora cactorum* in rootstocks M26 and MM106 (Rosati et al. 1990). The associated high cost of tissue culture is a major limitation in its large-scale exploitation. Automation of micropropagation protocols using bioreactors can be useful in this regard. Although problems are associated with automation, the use of immersion system is effective in apple shoot multiplication (Dobránszki and Teixeira da Silva 2010).

The presence of antibiotics/herbicides resistance marker gene and foreign DNA in transgenic plants adds to the concern regarding general acceptability, as they may pose a threat to the consumer's health and safety of the environment. The *nptII* gene encoding neomycin phosphotransferase II and phosphinothricin acetyltransferase encoding *bar* gene, which impart resistance against aminoglycoside antibiotics (such as genticin, kanamycin and neomycin) and herbicides (L-phosphinotricin, glyphosinate and bialaphos), respectively, were extensively used as selectable markers in apple transgenics (Degenhardt and Szankowski 2006). Alternatives of antibiotics resistance marker or raising marker-free plants are being explored with the hope of better acceptability among consumers. The *E. coli manA*, which encodes phosphomannose isomerase, was developed as an efficient positive selection marker system in apple, conferring transgenic plants an ability to grow on mannose as carbon source (Degenhardt and Szankowski 2006; Degenhardt et al. 2007). The *Eutypa lata* eutypine detoxifying gene (*Vr-ERE*; Chevreau et al. 2007) and the *galT* (galactose/UDP-glucose:galactose-1-phosphate uridyl-transferase) system (Degenhardt et al. 2007) are also being standardized as alternative selectable markers. Marker gene excision either by homologous recombination (such as FLP-FRT/Cre-lox system) or Ac/Ds transposon systems is being explored to raise marker-free apple plants. Schaart et al. 2004 described a recombination-mediated strategy using dexamethasone (DEX)-inducible plant-adapted recombinase and bifunctional selectable marker *codA-nptII* to raise marker-free strawberry and apple cultivar Elstar, although the data for apple were not presented. Recently, Malnoy et al. (2010) raised marker-free M26 and Galaxy using MDTT (markerless DNA transformation technology) protocol. However, the success of this technique depended on the availability of high efficient transformation system.

Recently, the concepts of intragenic and cisgenic plants have been explored, wherein the gene coding, and promoter and terminator sequences derived from the same crop or from sexually compatible species have been used to generate designer crops with more acceptability (Jacobsen and Schouten 2009; Rommens et al. 2007). However for this, the indigenous genes of apple and their regulatory sequences need to be characterized. Genes controlling fruit quality, shelf storage life, fruit softening and ripening are being characterized (Newcomb et al. 2006; Seo and Kim

2009; Wang et al. 2009). Apple genes imparting disease resistance are also being studied and one of such gene (*Vf*) along with its regulatory sequence had been used to develop cisgenic scab-resistant apple (Szankowski et al. 2009; Joshi et al. 2009; Malnoy et al. 2008). However, it is yet too early to comment on the potential of cisgenic plant and its acceptability. Efforts have also been initiated to understand the transcriptome associated with different developmental stages, and biotic and abiotic stresses in apple. Availability of microarray platform and advancement of next-generation sequencing technology would facilitate such initiatives. Due to progress in apple genome sequencing, the draft genome sequence is expected (Velasco et al. 2009). Availability of genome sequence and its proper annotation would help in isolating genes and their utilization for apple improvement. It may also facilitate the development of intragenic vector with all the DNA sequences derived from apple. This is an emerging concept that is being used to prevent the linkage drag of vector backbone in the progenies. Conner et al. (2007) have already identified some T-DNA-like sequences (P-DNAs: plant-derived DNAs) in apple; however, it is yet a long way to go before such an alternative becomes realistic in apple.

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